INFLUENCE OF THE INTRINSIC PROPERTIES OF FOOD ON THERMAL INACTIVATION OF SPORES OF NONPROTEOLYTIC *CLOSTRIDIUM BOTULINUM*: DEVELOPMENT OF A PREDICTIVE MODEL[1]

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ABSTRACT

The effects and interactions of heating temperature (70-90C), pH (5-6.5), sodium chloride (0-3%), and sodium pyrophosphate (0-0.3%) on the heat resistance of a six strain mixture of spores of nonproteolytic Clostridium botulinum *type B and type E in turkey were examined. Thermal death times were determined in submerged vials heated using a water bath. Heated spores were recovered on Reinforced Clostridial Medium (RCM) supplemented with lysozyme (10 μg/ml). Decimal reduction times (D-values) were calculated by fitting a survival model to the data with a curve fitting program. The D-values were analyzed by second order response surface regression for temperature, pH, salt (sodium chloride) and sodium pyrophosphate levels. The four variables interacted to effect the inactivation of spores. Confidence intervals (95%) predicted heat resistance of spores in turkey. The data suggest that the effect of reduced pH in increasing the inactivation was more pronounced at high temperatures and may provide an adequate degree of protection from nonproteolytic* C. botulinum *spores in minimally processed foods, particularly if employed in conjunction with combinations of salt and sodium pyrophosphate.*

INTRODUCTION

The consumer demand for fresh, high-quality, low salt, convenience meals with minimal preparation time has led to a new generation of precooked, extended shelf-life, refrigerated foods. These "cook/chill" foods receive a mild heat treatment (pasteurization) that primarily destroys the vegetative cells of spoilage and pathogenic bacteria; however, potentially pathogenic strains of the foodborne spore-forming bacteria can survive the thermal process. Although the market for such products is expanding, there has been continued concern regarding survival and growth of nonproteolytic *Clostridium botulinum*, a causative agent of the neuroparalytic disease, botulism.

Strains of *C. botulinum* have been classified into four groups; those that are of primary importance to food safety are group I strains (*C. botulinum* types A, B, and F) and nonproteolytic, group II (*C. botulinum* types B, E, and F) strains. While proteolytic type A and B strains of *C. botulinum* are more tolerant to environmental stresses, produce highly heat-resistant spores (Lynt *et al.* 1982) and have a minimal growth temperature of 10C (Smelt and Haas 1978), nonproteolytic *C. botulinum* strains are less tolerant to stresses, form considerably less heat resistant spores (Lynt *et al.* 1982; Scott and Bernard 1982) and can grow and produce toxin at temperatures as low as 3.3C (Solomon *et al.* 1977; Solomon *et al.* 1982). Consequently, the greatest food safety concern in cook/chill type foods is the growth of non-proteolytic *C. botulinum*; spores of those strains that survive the thermal process would pose a botulism hazard even under proper refrigeration temperatures if a secondary barrier is not present. Thus, without additional barriers heat processing must be sufficient to destroy the nonproteolytic *C. botulinum* spores if the food is to be safe.

Due to concerns about the health impact of the use of salt (sodium chloride), there is an increased demand to reduce the salt levels of meat products. The primary functions of salt in meat products are to: solubilize muscle proteins to assist in binding meat, moisture and fat; serve as a flavoring agent; and inhibit growth of foodborne pathogens. Efforts to reduce salt levels have resulted in increased use of phosphates to assure microbiological safety (Kijowski and Mast 1988). Choi *et al.* (1987) reported that salt levels could be reduced from 3% to 1.5% in frankfurters without processing or storage difficulties when a 0.5% blend of phosphates was added.

Changes in the intrinsic properties of the food products, primarily pH and salt content are known to affect the ability of spores to survive thermal processing in addition to their genotype. Sufficient evidence exists to document that the influence of environmental parameters in foods is even

greater than the inherent genetic factors (Put and Aalbersberg 1967). The influence of salt levels or pH of the heating menstruum on thermal inactivation of spores has been studied by many investigators (Montville and Sapers 1981; Cameron *et al.* 1980; Feeherry 1987; Lowik and Anema 1972; Briggs and Yazdany 1970; Cook and Gilbert 1969; Pivnick and Thacker 1970; Roberts *et al.* 1966; Juneja and Eblen 1994). Bacteria usually have their maximum heat resistance at neutral pH values. Spore sensitivity to heat is increased as the pH becomes acidic. Inclusion of salt in the heating menstruum results in reduced water activity which should account for increased heat resistance (Pflug and Holcomb 1983). While the effect of salt in the heating menstruum on the heat resistance of spores has shown conflicting results, heat injured spores are known to be increasingly sensitive to salt in the recovery medium. These intrinsic properties of food often are not being considered when designing thermal processes. Useful information regarding interactive effects of pH, salt, etc. on spore survival during thermal processing should aid in thermal process design. Studies have not been conducted in a manner to identify the maximal effectiveness of these parameters in lowering the thermal resistance, and multiple factorial experiments are lacking in which their interactive effect on the thermal resistance of *C. botulinum* can be determined. The combined effects of mild heat treatment, pH, salt and phosphate levels required to provide an adequate degree of protection from nonproteolytic *C. botulinum* spores in minimally processed foods must be well defined to ensure that the heating step is lethal, while avoiding heating that negatively impacts product quality. Accordingly, our study was initiated to quantitatively assess the effects of combinations of temperature, salt, pH, and phosphate on the thermal inactivation of *C. botulinum* in turkey slurry to assist food processors in the design of processing times and temperatures that ensure safety against non-proteolytic *C. botulinum* in cook/chill foods.

MATERIALS AND METHODS

Strains and Preparation of Spore Suspensions

Spores of three nonproteolytic *C. botulinum* type B and three type E strains used in the study were obtained and maintained as previously described (Juneja *et al.* 1994). Lack of proteolysis was verified by the lack of ability of the strain to digest iron milk and/or cooked meat. The

toxigenic serotype was confirmed by the antigen neutralization mouse bioassay (FDA 1984). *C. botulinum* confirmation was based on Gram-reaction, cellular morphology, lipase, catalase, and oxidase activities. Spores of *C. botulinum* were produced in trypticase-peptone-glucose-yeast extract by the procedure described previously (Juneja *et al.* 1994). After the spore crop of each strain had been washed twice and resuspended in sterile distilled water, the spore suspensions were stored at 2C until needed. A spore cocktail containing equivalent numbers of spores from all six strains of *C. botulinum* was prepared immediately prior to experiments. This composite spore mixture (8 $\log_{10}$ spores/ml) was heat-shocked for 10 min at 60C prior to use.

Preparation of Turkey Slurry

Ground turkey was purchased from a local supermarket and frozen (-5C) until use. A known weight of turkey was aseptically transferred to a sterile Waring Blendor, mixed with an equal volume of sterile water and blended for 2 min to form a smooth paste. Salt (0.0 - 3.0%, w/v) and/or sodium pyrophosphate (0.0 - 0.3%,w/v) was added in the turkey slurry which was again blended for 2 min to ensure even distribution. The pH of the turkey was adjusted to 5.0 - 6.5 using 85% (w/w) lactic acid (Sigma) and determined using a combination electrode (Sensorex, semi-micro, A.H. Thomas, Philadelphia, PA) attached to an Orion model 601A pH meter.

Experimental Design

A fractional factorial design was employed to assess the effects and interactions of heating temperature (70, 75, 80, 85 and 90C), salt (0.0. 1.0, 1.5, 2.0, 3.0%), sodium pyrophosphate (0.0. 0.1, 0.15, 0.2, 0.3%), and pH (5.0, 5.5, 6.0, 6.25, 6.5). All 43 variable combinations were replicated twice, each performed in duplicate vials.

Thermal Treatment of Spore Suspensions

Turkey slurry containing salt (0.0 - 3.0%, w/v) and sodium pyrophosphate (0.0 - 0.3%,w/v) at various pH levels (5.0 - 6.5) was inoculated with heat-shocked nonproteolytic *C. botulinum* type B and type E spore cocktail to obtain an initial count of about 7 $\log_{10}$ spores/ml. Inoculated samples were blended in a sterile Waring Blendor for 2 min to ensure even

distribution of spores. Thermal inactivation was carried out at 70, 75, 80, 85 or 90C in sterile 25 x 95 mm screw cap vials filled to the top with turkey slurry (10 ml) as described previously (Juneja *et al.* 1994). Negative controls included 2 vials containing uninoculated turkey slurry. The temperature was continuously monitored by two thermocouples inserted at the center of two uninoculated vials. A Keithyl-Metrabyte data logger model DDL 4100 (Taunton, MA) connected to a microcomputer was used to record the thermocouple readings. The thermocouple signal was sampled every second, and the two readings were averaged to determine the vial internal temperature. The time of heat treatments was based on temperature studied and ranged from 15 min to 45 min. The "come up" times to reach 70 and 90C were approximately 4 min and 2 min, respectively. Come-up times were included as part of the total heating time when these were used to calculate the D-values. Once the mixture reached the target temperature, two vials were removed and designated as time 0. Thereafter, two vials for each replicate were removed at six designated time intervals; sampling frequency was based on the heating temperature. After heating, vials were plunged into a crushed-ice bath.

Recovery of Heated Spores

After heat treatment, the surviving population of spores was determined in the meat sample by spiral plating (Model D, Spiral Systems, Cincinnati, OH) selected dilutions in 0.1% peptone water (wt/vol) onto agar plates containing RCM supplemented with lysozyme (10 μg/ml). The plates were incubated anaerobically in a Gas Pak system (Baltimore Biological Laboratory, Cockeysville, MD) at 28C for 6 days for recovery of heat-injured spores. For each replicate experiment with duplicate vials, an average surviving spore count of four platings of each sampling point was used to determine the D-values.

Calculation of D-values and Z-values

The D-values (time for 10-fold reduction in viable spores) were determined by a survival equation that was fitted to the experimental data using a Gauss-Newton curve fitting program (ABACUS Software Program, ERRC, USDA, Philadelphia, PA). This equation, given below, was developed by Whiting (1993) and was derived from the logistic-based equation of Kamau *et al.* (1990).

$$N = No + LOG\left[\frac{F1(1+e^{-b1*tl})}{(1+e^{b1(t-tl)})} + \frac{(1-F1)(1+e^{-b2*tl})}{(1+e^{b2(t-tl)})}\right]$$

Where,

b1 = maximum σ_2 ecific death rate of major population

b2 = maximum specific death rate of subpopulation

F1 = fraction of initial population in major population

(1 - F1) = F2 = fraction of population in subpopulation

tl = shoulder or lag period

t = time

N = population (log CFU ml^{-1}) surviving at time = t

No = initial population (log CFU ml^{-1}) at time = 0

D value is given by D = 2.3/b for each population

The z-values were estimated from the absolute value of the inverse slope by computing the linear regression (Ostle and Mensing 1975) of $\log_{10}$ D-values versus heating temperatures using Lotus 1-2-3 Software (Lotus Development Corporation, Cambridge, MA).

Statistical Modeling

The D-values were transformed to the natural logarithm form and analyzed by second order response surface regression to develop a regression model for temperature, pH, salt and sodium pyrophosphate levels.

RESULTS AND DISCUSSION

The present study assessed the effects and interactions of temperature, pH, salt and sodium pyrophosphate levels to quantify in turkey the inactivation of nonproteolytic *C. botulinum* type B and type E strains. Reinforced clostridial medium supplemented with lysozyme was used for recovery because prior research indicated that it gave the maximum heat-injured spores recovery (Juneja *et al.* 1994). Based on a minimal root

mean square value, the thermal inactivation data could be fitted well to generate survivor curves.

The multiple regression equation for the $\log_e$ D-values yielded an R^2 value of 0.953 (adjusted to 0.954). This equation, given below, based on 43 unique combinations, can predict D-values/spore survival for changes in the parameter values in the range tested from any combination of four environmental factors.

$$\text{Log}_e \text{ D-value} = -9.9161 + 0.6159(\text{temp}) - 2.8600(\text{pH}) - 0.2190(\text{salt}) + 2.7424(\text{phos}) + 0.0240(\text{temp})(\text{pH}) - 0.0041(\text{temp})(\text{salt}) - 0.0611(\text{temp})(\text{phos}) + 0.0443(\text{pH})(\text{salt}) + 0.2937(\text{pH})(\text{phos}) - 0.2705(\text{salt})(\text{phos}) - 0.0053(\text{temp})^2 + 0.1074(\text{pH})^2 + 0.0564(\text{salt})^2 - 2.7678(\text{phos})^2$$

For the environmental variables temperature, pH, salt and phosphate levels, the D-values of nonproteolytic *C. botulinum* spores based on survivor curves generated using the logistic-based survival equation are given in Table 1. The fit between D-values of spores in ground turkey as predicted by the model and those observed experimentally is given in Fig. 1. Lower pH of the turkey tended to increase the sensitivity of spores to heat, and this effect was substantially more at higher temperatures (Fig. 2). In the presence of 1.5% salt and 0.15% phosphate in turkey, the observed D-values at 70C decreased from 38.6 min to 33.8 min as the pH of the turkey was decreased from 6.0 to 5; the value at 90C for pH 5 turkey was 66.7% of the value at pH 6 (Fig. 2). These results with respect to temperature dependence of the pH effect are in agreement with the data presented by Cameron *et al.* 1980, while markedly different from others (Xezones and Hutchings 1965; Santos *et al.* 1992; Brown and Thorpe 1978). Cameron *et al.* (1980) heated *C. sporogenes* spores in phosphate buffer and pea puree in the pH range of 5.0 to 7.0 and found that the pH effect was more pronounced at the higher processing temperatures (118.3 - 121C), confirming the present results. In contrast, Xezones and Hutchings (1965) studied the effect of pH ranging from 4.0 to 7.0 on the heat resistance of *C. botulinum* spores in unprocessed food products (macaroni creole, spaghetti with tomato sauce and cheese, spanish rice). They found that the pH influence was greatest at lower temperature (110C), but at 118.3C there was little difference between pH 6 and 7. In a study by Lowik and Anema (1972), when pH of the minced meat inoculated with *Clostridium sporogenes* spores was dropped from 6.0 to 4.8, the D-values decreased by 40% irrespective of the heating temperature. Discrepancies between these studies and ours are to be expected because pH effects on heat resistance depends upon strain (Odlaug and Pflug 1976), suspending menstruum (Cameron *et al.* 1980), and a_w (Smelt *et al.* 1977).

TABLE 1.

OBSERVED AND PREDICTED D-VALUES AT 70-90C OF NONPROTEOLYTIC *C. BOTULINUM* IN GROUND TURKEY, AT VARIOUS pH LEVELS (5.0-6.5) SUPPLEMENTED WITH SALT (0.0-3.0%, w/v) AND SODIUM PYROPHOSPHATE (0.0 - 0.3% w/v)

Temperature	pH	% NaCl	%Phosphate	D-value Observed	D-value Predicted (UL)[b]
70	5.00	0.0	0.00	45.1 (0.6)[a]	63.0
70	5.00	1.5	0.15	33.8 (1.7)	38.5
70	6.00	0.0	0.00	51.3 (2.4)	58.7
70	6.00	0.0	0.30	33.9 (0.2)	52.2
70	6.00	1.5	0.15	38.6 (1.4)	41.3
70	6.00	3.0	0.00	31.1 (2.9)	50.1
70	6.50	0.0	0.00	57.7 (2.1)	66.0
70	6.50	1.5	0.15	40.1 (1.6)	46.5
70	7.00	1.5	0.15	44.1 (3.0)	61.3
75	5.00	1.5	0.15	28.9 (0.5)	30.4
75	5.50	1.0	0.10	36.1 (2.2)	38.4
75	5.50	1.0	0.20	28.5 (1.9)	32.2
75	5.50	2.0	0.10	28.7 (0.8)	31.8
75	5.50	2.0	0.20	20.9 (1.2)	26.3
75	6.00	1.5	0.15	36.4 (0.8)	33.8
75	6.25	1.0	0.10	39. 1 (0.2)	42.3
75	6.25	1.0	0.20	32.9 (0.3)	38.6
75	6.25	2.0	0.10	38.7 (1.0)	38.7
75	6.25	2.0	0.20	30.4 (0.5)	33.6
80	5.00	1.5	0.15	13.5 (0.2)	16.9
80	6.00	0.0	0.15	32.1 (0.5)	36.1

Temperature	pH	% NaCl	%Phosphate	D-value Observed	D-value Predicted (UL)[b]
80	6.00	1.5	0.00	26.2 (04)	30.2
80	6.00	1.5	0.15	23.0 (1.4)	25.6
80	6.00	1.5	0.30	21.0 (0.1)	24.0
80	6.00	3.0	0.15	25.1 (1.2)	27.1
80	6.50	1.5	0.15	28.8 (1.0)	30.2
85	5.00	1.5	0.15	7.0 (0.2)	7.7
85	5.50	1.0	0.10	7.4 (0.1)	11.2
85	5.50	1.0	0.20	6.7 (0.0)	9.8
85	5.50	2.0	0.10	7.4 (0.0)	9.8
85	5.50	2.0	0.20	4.0 (0.1)	7.6
85	6.25	1.0	0.10	13.5 (0.4)	16.4
85	6.25	1.0	0.20	11.9 (0.2)	14.0
85	6.25	2.0	0.20	9.7 (0.4)	11.6
90	5.00	0.0	0.00	5.0 (0.0)	6.3
90	5.00	1.5	0.15	3.1 (0.0)	4.8
90	6.00	0.0	0.00	8.8 (0.0)	9.5
90	6.00	0.0	0.30	5.4 (0.1)	5.9
90	6.00	1.5	0.15	4.8 (0.1)	4.8
90	6.00	3.0	0.00	5.6 (0.1)	6.3
90	6.25	2.0	0.20	3.5 (0.1)	4.8
90	6.50	0.0	0.00	9.3 (0.2)	13.6
90	6.50	1.5	0.15	7.3 (0.1)	7.1

[a]Values represent means (± standard deviations) of 43 variable combinations, each replicated twice and performed in duplicate.

[b]The upper limit of confidence interval of predicted D-value.

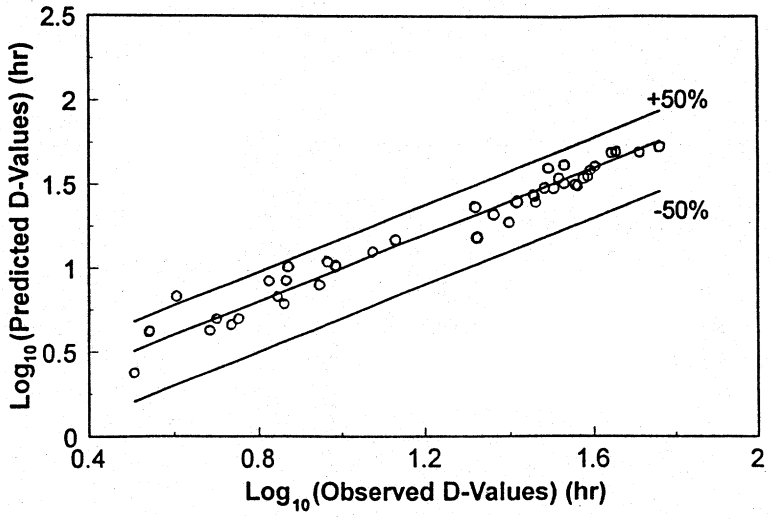

FIG. 1. AGREEMENT BETWEEN PREDICTED AND OBSERVED D-VALUES OF NON-PROTEOLYTIC *C. BOTULINUM* IN GROUND TURKEY.
The center line is the "line of identity" and the others represent ± 50% of the observed value.

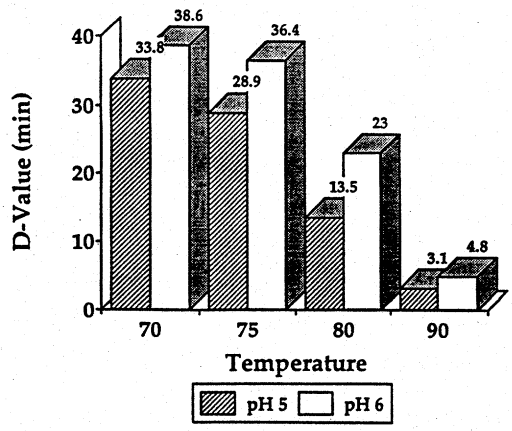

FIG. 2. EFFECT OF pH ON THE OBSERVED D-VALUES OF THREE NONPROTEOLYTIC *C. BOTULINUM* TYPE B AND THREE TYPE E STRAINS IN TURKEY SLURRY, THAT INCLUDED 1.5% SALT AND 0.15% SODIUM PYROPHOSPHATE, AT 70-90C

The predicted D-values for turkey samples containing no phosphate or added salt at 70C were 53.3 min and 49.5 min at pH 6.5 and 5, respectively (Fig. 3). While decreasing pH from 6.5 to 5 resulted in parallel decrease in predicted D-value by 7.1% at 70C (Fig. 3), the decrease was 35% at 80C and 54.1% at 90C (Data not shown).

Acidic conditions of the heating medium resulting in decreased heat resistance of spores may be attributed to single strand breaks in the DNA of spores (Sako *et al.* 1982; Northop and Slepecky 1967). Additionally, sufficient evidence exists to document that divalent cations (Ca^{++}, Mn^{++}, Mg^{++}) protect spore DNA from thermal injury (Alderton *et al.* 1976). Bender and Marquis (1985) suggested that the acidic conditions in the heating menstra strip the ions, creates instability of the nucleic acid, and sensitizes the spore DNA to heat.

Salt and/or phosphate addition increased heat sensitivity which was enhanced at low pH. For example, Fig. 3 depicts the effect of salt and/or phosphate on the predicted D-values at 70C. At the lower salt concentration (1%) in turkey, predicted D-values decreased (20.6%) from 49.5 min (turkey with no salt) to 39.3 min at pH 5 and from 53.3 min to 45.2 min (15.2% decrease) at pH 6.5. When turkey contained 3% salt, the D-values predicted were 34.8 min and 45.6 min at pH 5 and 6.5, respectively. It appears from these predicted D-values that at 70C, increasing salt levels can substantially decrease the D-values at low pH. Similar results were predicted at higher temperatures. Also, similar responses at all temperatures (70-90C) were observed when turkey was supplemented with phosphate (Fig. 3). For example, when turkey at pH 6 was supplemented with 0.3% phosphate, the observed D-values at 70C decreased by 34.0% (Table 1). The results agree with previous studies (Cook and Gilbert 1969; Briggs and Yazdany 1970) in which the heat resistance of *Bacillus stearothermophilus* spores was progressively reduced with increasing salt concentrations (2, 4 and 8% w/v) in the heating medium. A similar trend was noted for nonproteolytic *C. botulinum* type B spores in turkey slurry supplemented with >2% salt levels (Juneja and Eblen 1994). Spores exhibiting decreased heat resistance with increasing salt and phosphate concentration may be attributed to the toxic effect of salt leading to spore injury during heating.

A combination of salt and phosphate appeared to be more effective in decreasing the D-values, with greater impact at low turkey pH. An example of the interaction between salt and phosphate at two pH levels on the observed D-values is presented in Fig. 4. The D-values at 75C in turkey supplemented with 1% salt and 0.1% phosphate at pH 5.5 and 6.25 were 36.1 and 39.1, respectively. Addition of more salt and/or phosphate decreased the D-values. When turkey contained 2% salt and 0.2% phosphate, the observed D-values in turkey at pH 5.5 and 6.25 decreased by

42.1% and 22.1%, respectively. The response of the spores to both salt and phosphate levels at higher temperatures was similar.

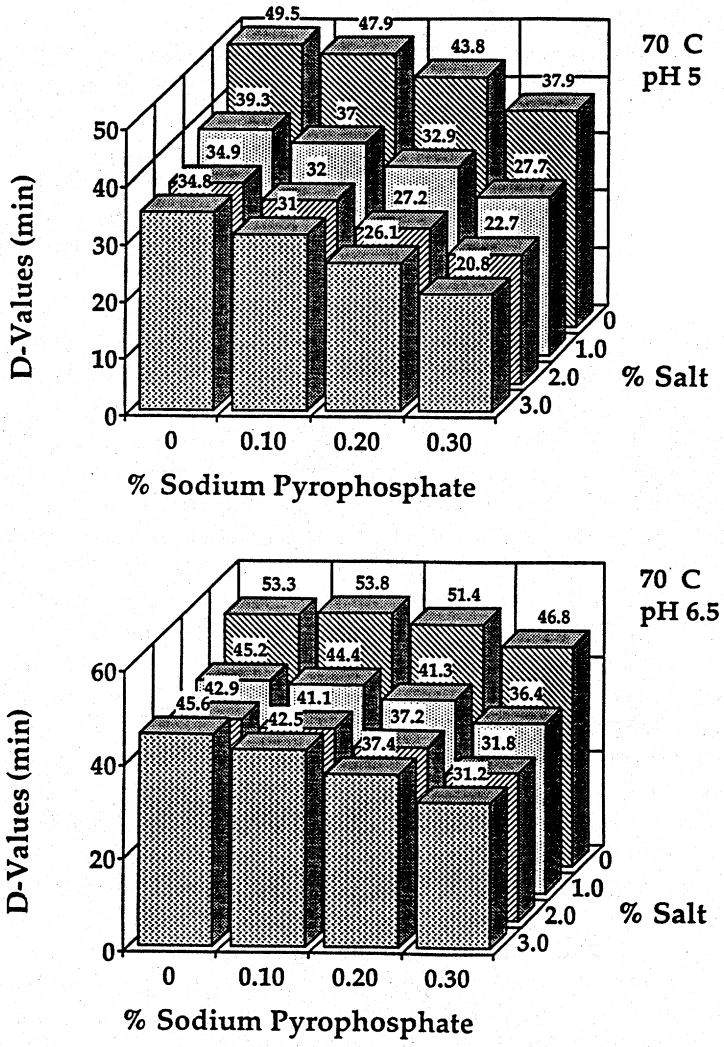

FIG. 3. EFFECTS AND INTERACTIONS OF TEMPERATURE, SALT, pH AND SODIUM PYROPHOSPHATE ON THE PREDICTED D-VALUES, AT 70C, OF THREE NONPROTEOLYTIC *C. BOTULINUM* TYPE B AND THREE TYPE E STRAINS IN TURKEY SLURRY AT pH 5 AND 6.5

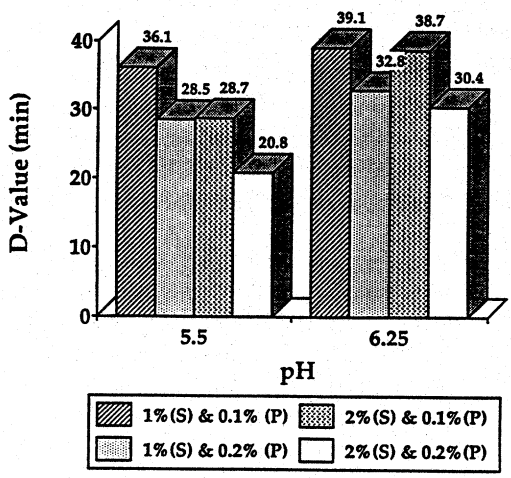

FIG. 4. EFFECTS AND INTERACTIONS OF SALT AND SODIUM PYROPHOSPHATE LEVELS ON THE OBSERVED D-VALUES, AT 75C, OF THREE NONPROTEOLYTIC *C. BOTULINUM* TYPE B AND THREE TYPE E STRAINS IN TURKEY SLURRY AT pH 5.5 AND 6.25

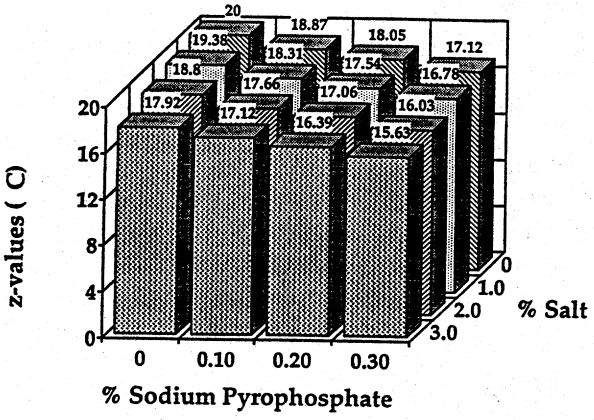

FIG. 5. THE Z-VALUES CALCULATED FROM PREDICTED D-VALUES OBTAINED IN TURKEY (pH 5) SUPPLEMENTED WITH 0.0 - 3.0% SALT AND/OR 0.0 - 0.3% SODIUM PYROPHOSPHATE

Figure 3 depicts the predictive relative impact of various levels of salt and phosphate in increasing the sensitivity of spores to heat. The z-value calculated from predicted D-values obtained in turkey (pH 5) supplemented with 1.0% salt and 0.1% sodium pyrophosphate was 18.3C (Fig. 5). Predicted D-values obtained in turkey with additional salt and/or phosphate resulted in lower z-values. The present study indicates that smaller changes in temperature are required to cause 90% reduction in D-value when a cocktail of spores are evaluated in turkey with increasing levels of salt and/or phosphate.

The present study presents an assessment and quantification of the effects and interactions of temperature, pH, salt and phosphate levels and indicates that the thermal inactivation of nonproteolytic *C. botulinum* type B spores is dependent on all four factors. Thermal resistance of spores can be lowered by combining these intrinsic factors. The multiple regression equation developed in this study can predict D-values for any combinations of temperature, salt, sodium pyrophosphate, and pH that are within the range of those tested. Using this predictive model food processors should be able to design thermal processes for the production of a safe food with extended shelf life without substantially adversely affecting the quality of the product.

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